Immunity, Vol. 14, 33–43, January, 2001, Copyright ©2001 by Cell Press

# Activation and Anergy in Bone Marrow B Cells of a Novel Immunoglobulin Transgenic Mouse that Is Both Hapten Specific and Autoreactive

**Robert J. Benschop,* Katja Aviszus,* Xianghua Zhang,* Tim Manser,† John C. Cambier,*‡§ and Lawrence J. Wysocki*‡§**

*Integrated Department of Immunology
National Jewish Medical and Research Center and
University of Colorado School of Medicine
Denver, Colorado 80206
†Kimmel Cancer Institute
Department of Microbiology and Immunology
Jefferson Medical College
Philadelphia, Pennsylvania 19107

## Summary

**Available evidence indicates that B cell tolerance is attained by receptor editing, anergy, or clonal deletion. Here, we describe a *p*-azophenylarsonate (Ars)-specific immunoglobulin transgenic mouse in which B cells become anergic as a consequence of cross-reaction with autoantigen in the bone marrow. Developing bone marrow B cells show no evidence of receptor editing but transiently upregulate activation markers and appear to undergo accelerated development. Mature B cells are present in normal numbers but are refractory to BCR-mediated induction of calcium mobilization, tyrosine phosphorylation, and antibody responses. Activation marker expression and acquisition of the anergic phenotype is prevented in bone marrow cultures by monovalent hapten. In this model, it appears that induction of anergy in B cells can be prevented by monovalent hapten competing with autoantigen for the binding site.**

## Introduction

B cell fate is precisely controlled by constitutive and antigen-driven transduction of antigen receptor (BCR) signals. While constitutive signals transduced by pro-BCR, pre-BCR, and BCR sustain development and survival in the B cell lineage, antigen aggregation of BCR enhances this signaling to initiate receptor editing, apoptosis, anergy, or the immune response (Benschop and Cambier, 1999). The nature of the antigen-induced response depends on the developmental stage of the responding cell, the avidity of the antigen-BCR interaction, and availability of helper T cell signals. This differential responsiveness to antigen plays a critical role in eliminating autoreactive B cells from the functional repertoire, resulting in immunologic tolerance.

Immature B cells are exquisitely sensitive to tolerance induction compared to their mature counterparts (Cambier et al., 1976; Metcalf and Klinman, 1976; Pike et al., 1982). It appears that multiple mechanisms are at play in the induction and maintenance of B cell tolerance to self. These mechanisms have been revealed largely from two immunoglobulin transgenic (Tg) models; the 3–83 μδ mouse, in which the low-affinity ($K_a = 2 \times 10^5\ M^{-1}$) BCR is specific for the cell-associated antigen H-2K$^{k/b}$ (reviewed by Nemazee, 2000; Nemazee et al., 2000), and the HyHEL10 (MD4) mouse, in which the BCR exhibits high affinity ($K_a \geq 2 \times 10^9\ M^{-1}$) for hen egg lysozyme (HEL) (reviewed by Healy and Goodnow, 1998). Distinct BCR-dependent tolerance mechanisms appear to be operative in the various stages of B cell development. Aggregation of BCR on immature B cells in the bone marrow by membrane-associated antigen induces receptor editing and clonal deletion (Nemazee and Burki, 1989; Hartley et al., 1991). Receptor editing involves activation of RAG genes and leads to expression of a BCR with altered antigen specificity (Gay et al., 1993; Tiegs et al., 1993). If this process does not eliminate autoreactivity before the cell reaches the transitional stage (mIgM$^{high}$mIgD$^{low/neg}$), BCR aggregation stimulates death by apoptosis (Carsetti et al., 1995; Norvell et al., 1995; Melamed et al., 1998). Binding of proteinaceous antigens (e.g., soluble HEL) to mature B cells in the periphery leads to antigen processing and upregulation of molecules such as CD86, in preparation for collaboration with T cells. If these interactions do not occur within a finite period, i.e., ~48 hr, B cells enter a state characterized by continued expression of receptors capable of binding antigen but incapable of signal transduction, known as anergy. Although these cells bind antigen, mIgM and mIgD are downregulated usually by ~90% and 50%, respectively, in the HEL model (Goodnow et al., 1988). These receptors fail to transduce signals leading to even the most proximal biochemical consequences studied, i.e., tyrosine phosphorylation of signaling intermediaries and induction of calcium mobilization and expression of CD86 and MHC class II (Goodnow et al., 1988; Cooke et al., 1994; Vilen et al., 1997). However, these anergic cells remain responsive to CD40-mediated signals (Cooke et al., 1994; Eris et al., 1994). Based on the selective ability of cell membrane-associated and soluble HEL to induce clonal deletion and anergy, respectively (Goodnow et al., 1988; Hartley et al., 1991), it has been suggested that antigen avidity is a key determinant of the mode of B cell tolerance induction. However, it is clear that B cell developmental stage codetermines the fate of B cells encountering antigen (Carsetti et al., 1995; Norvell et al., 1995; Benschop et al., 1999). The relative importance and interplay of each of these parameters is unclear.

In this report, we describe the properties of an immunoglobulin (μδ/κ) Tg mouse expressing a canonical anti-Ars BCR. In contrast to the immunoglobulin Tg models described, hapten-specific antibodies offer the freedom to control avidity and affinity. The response elicited by *p*-azophenylarsonate (Ars) in A/J mice is a classical example of a hapten-specific immune reaction (Kuettner et al., 1972). It consistently gives rise to a memory immune response in which approximately ~50% of the antibodies can be described as "canonical," and accordingly has been the subject of numerous investigations of

‡To whom correspondence should be addressed (e-mail: cambierj@njc.org [J. C. C.], wysockil@njc.org [L. J. W.]).
§These authors contributed equally to this work.

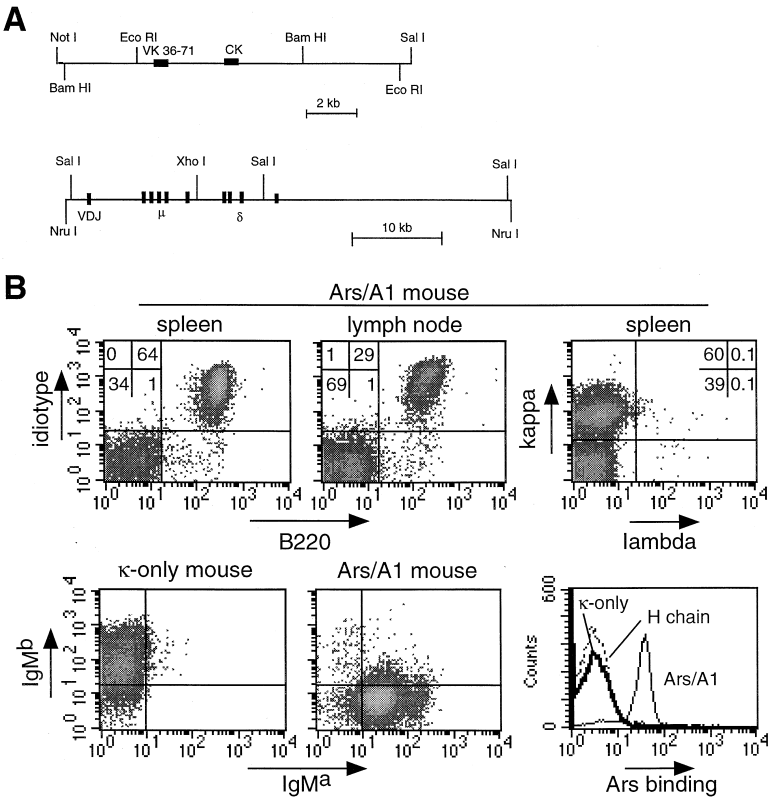


Figure 1. Generation and Analysis of Ars/A1 Tg Mice

(A) Schematic representation of the 36–71 L chain and 36–65 H chain constructs used to generate μδ Ars-specific Ig-Tg mice (Ars/A1).

(B) Flow cytometric analysis of peripheral lymphocytes from Ars/A1, κ-only, and H chain–only Tg mice. Spleen and lymph node (for Ars/A1) were analyzed using B220 and an anti-idiotypic antibody (E4). Efficiency of allelic exclusion was determined by analysis of L chain expression in Ars/A1 mice (κ versus λ), as well as by IgM-allotype and antigen binding capability (gated on $B220^+$ cells) in the spleen. Numbers represent percentage of positive cells in each quadrant.

memory B cell development (Manser et al., 1998; Wysocki et al., 1998). Canonical anti-Ars antibodies are highly uniform in structure. They are encoded by one combination of VH, D, JH, Vκ, and Jκ gene segments. An arginine codon forms the Vκ-Jκ junction, and the D region is uniform in length. Prior to somatic mutagenesis, canonical antibodies only differ at codons 100 and 107 at the VH-D and D-JH boundaries, respectively. Surprisingly, this Ars-specific Tg BCR in which the κ chain has multiple mutations is cross-reactive with an undefined self-antigen that induces an activated/anergic phenotype in developing bone marrow B cells. This phenotype can be modulated by the monovalent hapten Ars-tyrosine, apparently by competing for the BCR binding site. This model provides the ability to explore tolerance with powerful approaches historically applied to anti-hapten immune responses.

## Results

### Transgene Expression and Allelic Exclusion in Ars/A1 Tg Mice

Two lines of Tg mice were generated expressing an unmutated H chain V region gene from hybridoma 36–65 in the context of μ and δ or the heavily mutated κ light chain gene from hybridoma 36–71 (Figure 1A). Interbreeding led to the generation of mice (termed Ars/A1 mice) that expressed both H + L transgenes. These animals contained normal numbers of B220-positive cells in the spleen and lymph nodes.

Approximately 95% of B cells stained brightly with the E4 anti-idiotype (Figure 1B), which requires pairing of respective H and κ variable region domains for activity (Leo et al., 1985; Liu et al., 1996). Furthermore, analyses using allotype-specific reagents demonstrated good expression of the $\mu^a$ Tg receptor and nearly complete allelic exclusion (>95%). Virtually no λ light chain–expressing B cells were detected in Ars/A1 mice (Figure 1B). Consistent with anti-idiotype staining, Ars/A1 B cells, but not B cells from κ-only or H chain–only Tg mice, stained with a biotinylated conjugate of $Ars_8$-BSA (Figure 1B), indicating that both H and L chains are required to generate an Ars-specific BCR. Together, these data demonstrate successful expression of both transgenes and suggest good allelic exclusion.

### Ars/A1 Mice Fail to Mount a Primary Immune Response

To assess their ability to mount a primary immune response, Ars/A1 mice were immunized with an Ars conjugate of KLH ($Ars_{14}$-KLH) emulsified in incomplete Freund's adjuvant. The immune response to Ars was examined serologically and by immunohistochemistry. Surprisingly, there was virtually no Ars-specific antibody in the sera of preimmune mice and negligible Ars-specific IgM titers at d13 postimmunization (Figure 2A). In one Ars/A1 mouse (out of eight), a low Ars-specific titer was observed preimmunization, which did not increase significantly upon immunization. In contrast, significant anti-Ars IgM titers were obtained from sera of immunized κ-light chain–only Tg littermates (Figure 2A). In addition, a rapid class-switch to IgG occurred in the κ-only Tg mice. Finally, similar results were obtained using complete Freund's adjuvant (data not shown). Histochemical

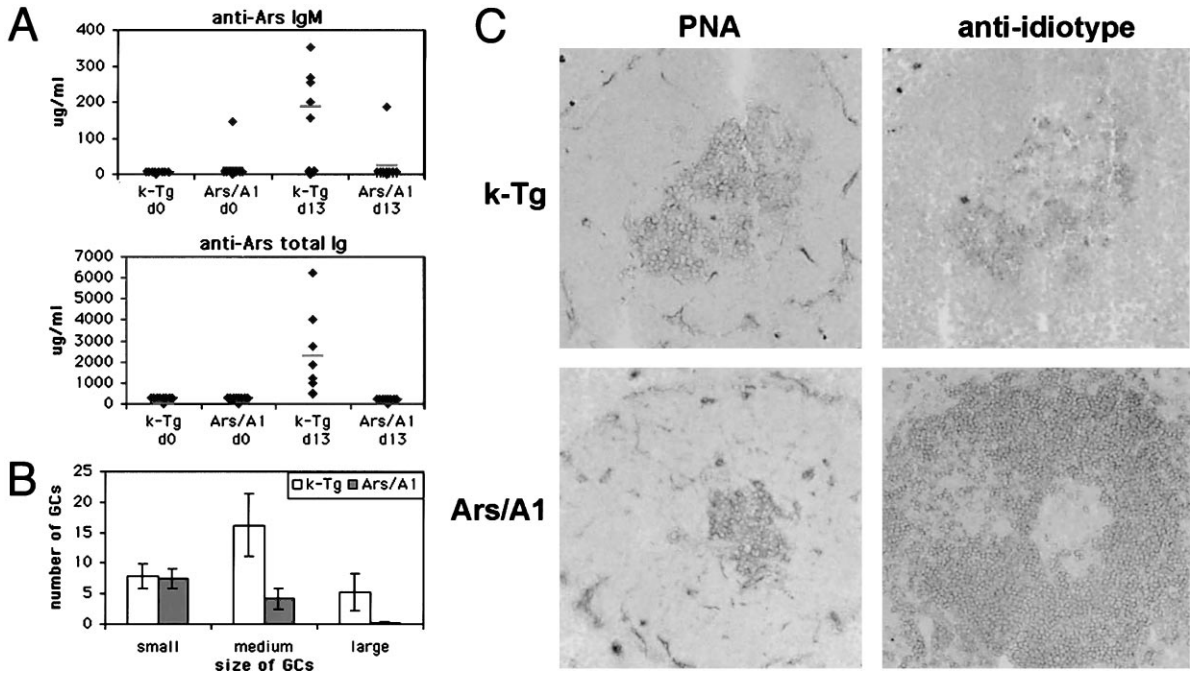


Figure 2. Ars/A1 Tg Mice Fail to Mount an Immune Response to Ars

Ars/A1 and κ-Tg mice were immunized with $Ars_{14}$-KLH.

(A) Serum was analyzed for the presence of Ars-specific IgM or total immunoglobulin before (day 0) and after immunization (day 13). Values for individual mice are shown (eight per group) with indicated mean values.

(B) Analysis of the number and size (arbitrary units, see Experimental Procedures) of GC following immunization (mean ± SEM).

(C) Immunohistochemistry of Ars/A1 and κ-Tg spleens postimmunization (day 13). GCs are visualized using PNA stain; successive sections were stained for anti-idiotype expression. In Ars/A1 cells, B cells surrounding the GC are idiotype positive, whereas cells in the GC appear negative. In contrast, in κ-Tg mice, idiotype-positive cells are found only in the GC. In agreement with receptor downregulation in GCs, idiotype staining was less prominent in κ-Tg mice compared to idiotype-positive cells in Ars/A1.

analyses revealed that germinal centers (GC) were smaller and fewer in Ars/A1 than in κ-only Tg mice (Figures 2B and 2C), and the few GC that were seen in Ars/A1 mice appeared idiotype negative (Figure 2C). Collectively, these results indicate that despite expression of Ars-specific antigen receptors capable of binding antigen, Ars/A1 B cells are immunologically compromised, appearing anergic.

### Accelerated Peripheral Development and Activation Marker Expression on Ars/A1 B Cells In Vivo

Although initial analyses of spleen and lymph nodes of Ars/A1 animals did not indicate gross abnormalities with respect to the peripheral B cell compartment, the serological and histological observations prompted us to analyze the phenotype of mature and immature B cells in these mice more carefully. We compared Ars/A1 and κ-only Tg lymphocyte expression of cell surface molecules indicative of activation status. No differences were observed with respect to B cell expression of B220, CD19, CD62L, and CD22 (data not shown). In contrast, levels of IgD were higher in Ars/A1 B cells while IgM expression was reduced by 40%–60% compared to cells from κ-only Tg littermates (Figure 3A). In addition, the number of transitional and marginal zone B cells ($IgM^{high}IgD^{low/neg}$) in the spleen was ~10 fold reduced in Ars/A1 spleens (Figure 3A). This was substantiated by the virtual absence of $CD23^{neg}$ B cells in Ars/A1 spleens (data not shown). Analysis of CD21/35 expression demonstrated an ~6 fold reduction in the number of transitional T2 B cells (Loder et al., 1999) in Ars/A1 spleen compared to κ-only Tg animals (Figure 3A). Importantly, CD80 was upregulated on mature Ars/A1 B cells, whereas CD86 expression appeared normal. This staining pattern suggests accelerated peripheral development in Ars/A1 mice, possibly as a consequence of B cell signaling by an endogenous antigen.

Subsequent analysis of bone marrow from Ars/A1 and κ-only Tg animals revealed elevated levels of CD80 and CD86 especially on transitional ($IgD^{low}$) B cells (Figure 3B). Most pre- and immature B cells (lower quadrants) were negative for all activation markers. CD80 and especially CD86 expression levels were lower on the recirculating mature B cells ($IgD^{high}$) found in the bone marrow compared to transitional B cells, consistent with the transience of induced expression of these markers (Hathcock et al., 1994). The expression of CD69 was upregulated similarly to CD80, i.e., elevated expression in the transitional B cells in the bone marrow with lower levels in the spleen (data not shown).

These results suggest that in Ars/A1 mice an autoantigen present in the bone marrow stimulates transient upregulation of molecules that are important in T-B collaboration. Apparently, this stimulation is qualitatively or quantitatively inadequate to induce tolerance by receptor editing or clonal deletion mechanisms because

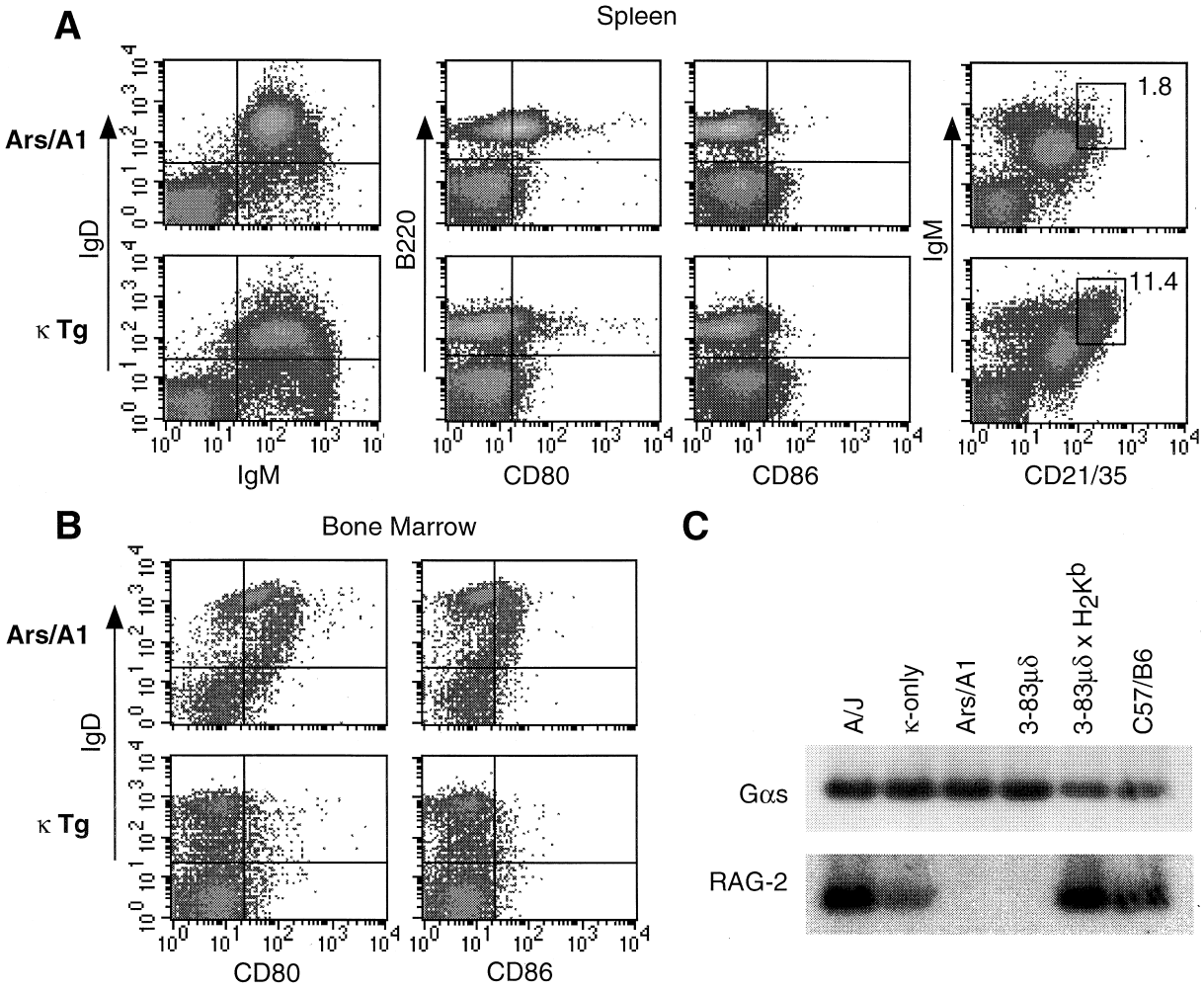


Figure 3. Activated Phenotype of Splenic and Bone Marrow B Cells in Ars/A1 Mice

Spleen (A) and bone marrow (B) from Ars/A1 and κ-only Tg littermates were analyzed by flow cytometry. Analyses of spleen cells show live ($7AAD^{neg}$) cells, analyzed for IgM, IgD, CD21/CD35, CD80, CD86, and B220. Bone marrow diagrams show live ($7AAD^{neg}$) $B220^{+}$ cells, analyzed for CD80 and CD86 by IgD to separate pre- and immature B cells (lower quadrants) from transitional ($IgD^{low}$) and mature circulating ($IgD^{high}$) B cells. (C) Expression of RAG-2 and GαS (control) was analyzed in immediately ex vivo bone marrow cells ($5 \times 10^6$) from non-Tg A/J and C57/B6 mice, κ-only Tg, Ars/A1 Tg, 3–83μδ Tg, and 3–83μδ Tg mice bred onto antigen ($H_2K^b$)-expressing C57/B6 using RT-PCR.

peripheral B cells are normal in number and continue to express the Tg receptor (Figure 1). To further test this interpretation, RAG2 expression was analyzed in bone marrow by RT-PCR. In models of autoantigen-induced receptor editing, BCR-mediated stimulation of bone marrow cells results in enhanced expression of RAG2 (Melamed et al., 1997; Benschop et al., 1999). In contrast to control A/J and κ-only Tg bone marrow, no RAG2 expression was observed in ex vivo Ars/A1 bone marrow cells (Figure 3C). The absence of RAG2 in Ars/A1 bone marrow is typical of a nontolerant immunoglobulin Tg animal (see the 3–83 μδ control; Figure 3C). In contrast, RAG2 is readily detected in 3–83 μδ Tg mice bred onto antigen-expressing mice (C57/B6), the model for receptor editing (Tiegs et al., 1993). These data demonstrate that the signal given by the autoantigen is quantitatively insufficient or qualitatively inappropriate to induce receptor editing in immature B cells. Instead, this interaction induces transient expression of molecules relevant for T-B collaboration and permits accelerated development of a peripheral B cell compartment with an intact autoreactive BCR.

### Blocking Activation of Bone Marrow B Cells with Free Hapten

To explore whether the activated phenotype of Ars/A1 B cells is a consequence of antigen receptor signaling, we assessed the ability of monovalent hapten (Ars-tyrosine) to modulate activation of in vitro propagated Ars/A1 bone marrow B cells. We hypothesized that if the effects seen are due to autoantigen binding to the BCR, monovalent hapten may block them. Bone marrow B cells of Ars/A1 mice developed and expanded normally during the 6 days culture in IL-7 (Benschop et al., 1999). Cells that developed in medium with IL-7 alone were capable of binding $Ars_8$-BSA and displayed upregulation of CD69 and CD80 (Figure 4A, top row), consistent with the in vivo phenotype. However, when propagated in

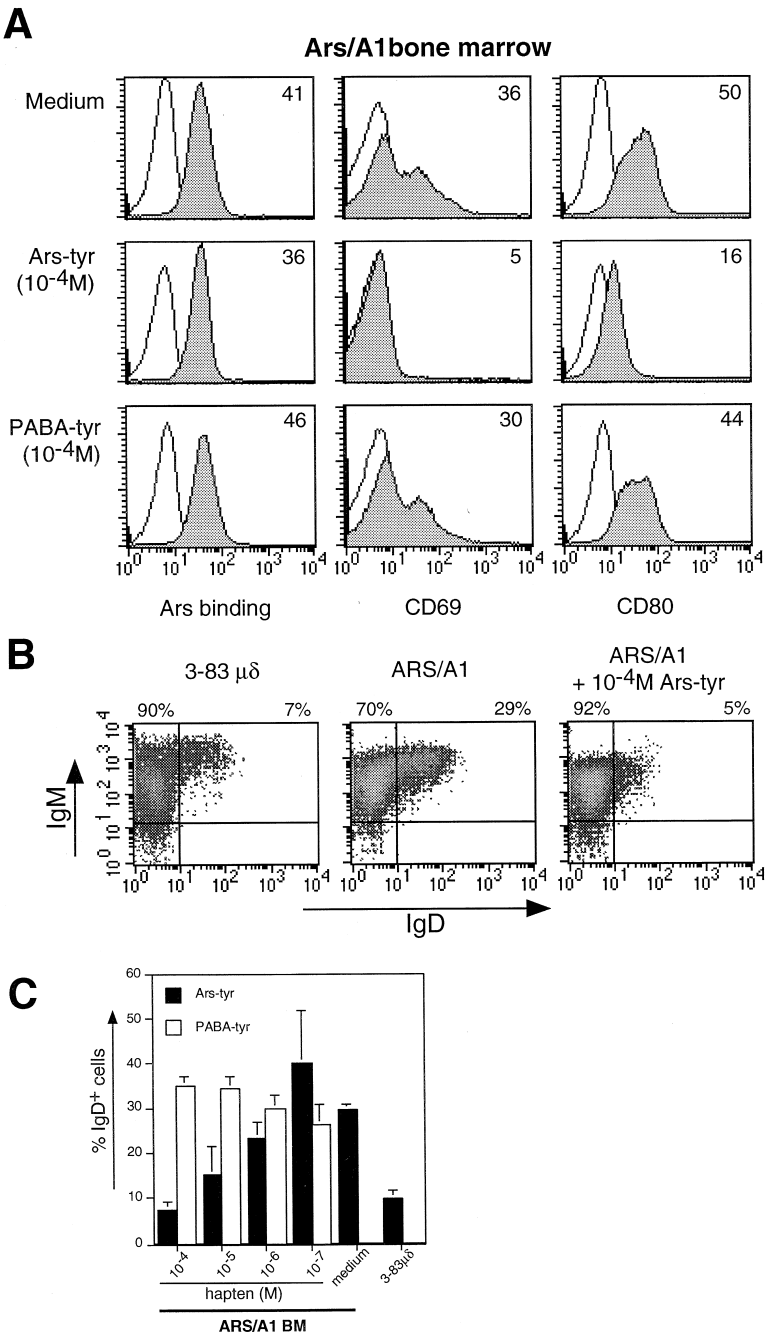


Figure 4. Activation of Ars/A1 Bone Marrow B Cells Blocked by Monovalent Ars-Tyrosine

(A) Bone marrow cells from Ars/A1 mice were cultured in IL-7 containing medium with or without Ars-tyrosine (Ars-tyr; $10^{-4}$ M) or PABA-tyrosine (PABA-tyr; $10^{-4}$ M). After 6 days of culture, cells were analyzed by flow cytometry for ability to bind antigen ($Ars_8$-BSA) and expression of activation markers (CD69 and CD80); histograms of live B cells ($7AAD^{neg}B220^{+}$) are shown (unstained cells are shown in white; mean fluorescence 4 to 7) with mean fluorescence of the gray curves indicated.

(B) Analysis of IgM and IgD in 6 day bone marrow cultures of Ars/A1 (with or without $10^{-4}$ M Ars-tyr) and 3–83 μδ Tg mice. The percentage of positive cells in the upper left and right quadrants are indicated.

(C) Dose-dependent effect of Ars-tyr on the frequency of $IgD^{+}$ cells in 6 day Ars/A1 and 3–83 μδ bone marrow cultures (black bars; open bars represent effect of the addition of PABA-tyr). Mean ± SEM of two independent experiments are shown.

the presence of monovalent Ars-tyrosine, the emerging B cells were CD69 negative and CD80 expression was ~3-fold lower than in the absence of Ars-tyrosine (Figure 4A, middle row). In contrast, a control hapten, PABA-tyrosine, had no effect on expression of these activation markers (Figure 4A, bottom row). All three culture conditions yielded equivalent numbers of B cells, and B cells from all three cultures bound $Ars_8$-BSA equally well (left column). These data strongly support the possibility that an endogenous antigen induces B cell activation in vivo and in bone marrow cultures of Ars/A1 mice. It is noteworthy that the affinity of the Ars/A1 antibody for Ars is only ~2.5 × $10^5$ $M^{-1}$ (Sharon, 1990). Disruption by monovalent hapten suggests that the autoantigen binds the Tg BCR with a low affinity.

The unusually high expression of mIgD by peripheral B cells suggested that B cells in these animals are, in a sense, hypermature. To explore the role of autoantigen in generation of this phenotype, we investigated whether there was any evidence for accelerated B cell development in IL-7-driven bone marrow cultures. While in non-Tg animals cells accumulate at the late pre-B cell stage ($IgM^{neg}IgD^{neg}$) (Rolink et al., 1991), the presence of an immunoglobulin Tg allows cells to become IgM positive under these culture conditions while largely remaining IgD negative (Melamed et al., 1997; Benschop et al.,

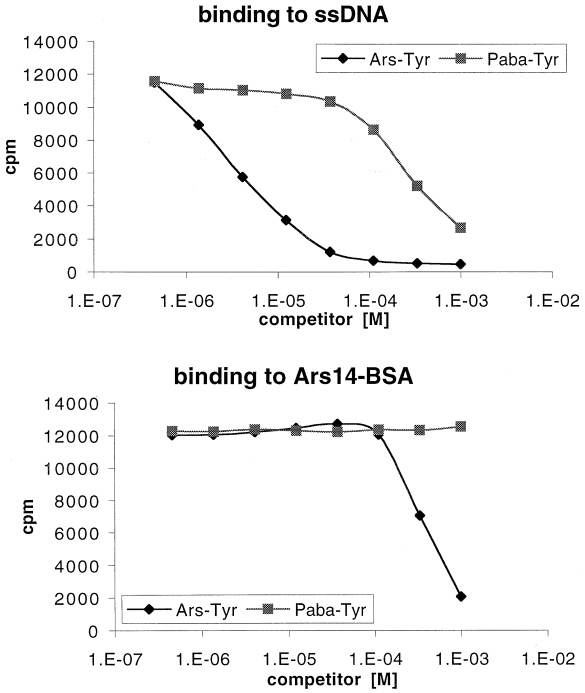


Figure 5. Binding of ssDNA by the Ars/A1 Antibody

The ability of the Ars/A1 receptor to bind ssDNA and $Ars_{14}$-BSA was tested in competition binding assays using an IgG2b correlate. Increasing concentrations of competitor (monovalent Ars-Tyr or PABA-Tyr) were added to the wells, and binding antibody was detected using a monoclonal $^{125}$I-labeled anti-κ. Standard deviation was <1%. Background counts for binding to polylysine (1054 cpm) in A and BSA in B (364 cpm) were subtracted.

1999). Although numbers of Ars/A1 and 3–83 μδ cells were comparable following a 6 day culture, we observed an increase in the percentage of $IgD^+$ cells in Ars/A1 bone marrow cultures (Figure 4B). The percentage of $IgD^+$ cells in a nontolerant bone marrow culture (here 3–83 μδ) was 5%–7%, but an increase to ~30% was observed in bone marrow cultures from Ars/A1 mice (Figure 4B). Interestingly, we observed a correlation between IgD and activation marker expression in these cultures (data not shown) as would have been expected based on the results obtained using ex vivo bone marrow (Figure 3B). The increased frequency of $IgD^+$ cells was not observed when $10^{-4}$ M Ars-tyrosine was present (Figure 4B) and was directly correlated with the amount of Ars-tyrosine present in the cultures (Figure 4C). The control hapten PABA-tyrosine did not block increased frequency of $IgD^+$ cells (Figure 4C). Addition of hapten to 3–83 μδ bone marrow cultures did not alter their growth characteristics or phenotypic appearance (data not shown). These results suggest that an autoantigen either induces an advanced state of differentiation or increases longevity or proliferation of cells that have acquired this phenotype.

### Affinity of Ars/A1 Tg Receptor for ssDNA

In an effort to identify the autoantigen reactive with the Ars/A1 receptor, we tested a corresponding IgG2b isotypic form of this antibody for binding to a panel of autoantigens in solid-phase radioimmunoassay. A significant interaction with ssDNA was observed (Figure 5). This interaction was binding-site specific, as revealed by competition with monovalent Ars-tyrosine. 100-fold more PABA-tyrosine, which has a much reduced affinity for the canonical binding site (Wysocki and Sato, 1981), was required to achieve the same level of competition. We can infer that the affinity of the bond between the antibody and ssDNA was low because the concentration of Ars-tyrosine required to inhibit binding to ssDNA was ~100-fold less than required to inhibit binding to $Ars_{14}$-BSA. Consistent with the possibility that ssDNA is the responsible autoantigen is the fact that $10^{-4}$ M Ars-tyrosine completely blocks both the binding to the Ars/A1 antibody and the response of Ars/A1 B cells to antigen. Whether ssDNA is the actual autoantigen responsible for B cell activation in this Tg mouse is uncertain, however, because antibodies that bind DNA often have secondary specificities (Diamond and Scharff, 1984; Retter et al., 1996; Santulli-Marotto et al., 1998; Takeda et al., 1999; Eivazova et al., 2000).

### Defective BCR-Mediated Signal Transduction in Ars/A1 B Cells

Anergic B cells have been analyzed extensively in the HEL/anti-HEL tolerance model (Goodnow et al., 1988). In that model, as well as in similar lymphoma models (Vilen et al., 1997), B cells are refractory to BCR stimulation as measured by a failure to induce receptor tyrosine phosphorylation and kinase activation and by elevated basal levels of Erk1 and Erk2 phosphorylation. In addition, anergic B cells have elevated basal intracellular free calcium concentrations ($[Ca^{2+}]_i$) as compared to nonanergic counterparts and fail to mobilize $Ca^{2+}$ following receptor aggregation (Cooke et al., 1994; Healy et al., 1997). We therefore analyzed these indicators of B cell anergy in mature splenic B cells from Ars/A1 animals and κ-only Tg littermates.

Splenic B cells were stimulated using anti-μ, lysed, and analyzed by immunoblotting with anti-phosphotyrosine. B cells from Ars/A1 mice displayed a markedly reduced response to BCR ligation compared to B cells from the κ-only Tg mice (Figure 6A). In addition, levels of Erk1 and Erk2 phosphorylation were elevated in unstimulated Ars/A1 B cells (Figure 6B). Anti-μ antibodies stimulated only modest increases in Erk phosphorylation in Ars/A1 cells compared to the response of κ-only Tg mice. Consistent with findings in the anti-HEL system (Healy et al., 1997), antigen-stimulated Erk phosphorylation in the anergic Ars/A1 cells (Figure 6B). $[Ca^{2+}]_i$ was analyzed in indo-1AM-loaded cells. Baseline $[Ca^{2+}]_i$ was clearly elevated in the majority of Ars/A1 B cells, suggesting continuous low-level stimulation or long memory of the BCR aggregation that led to receptor desensitization (Figure 6C). Minimal additional calcium mobilization was observed in Ars/A1 B cells following receptor aggregation (Figure 6D). Consistent with a nontolerant mature phenotype, B cells from κ-only Tg mice displayed low basal (70–90 nM) $[Ca^{2+}]_i$ and mounted a strong response to anti-μ. The observations made in the κ-only Tg mice were similar to those observed in B cells from non-Tg A/J animals or B cells from nontolerant 3–83 μδ mice (data not shown). Together, the data demonstrate that mature B cells from Ars/A1 Tg mice display the hallmarks of B cells rendered anergic by a receptor desensitization mechanism.

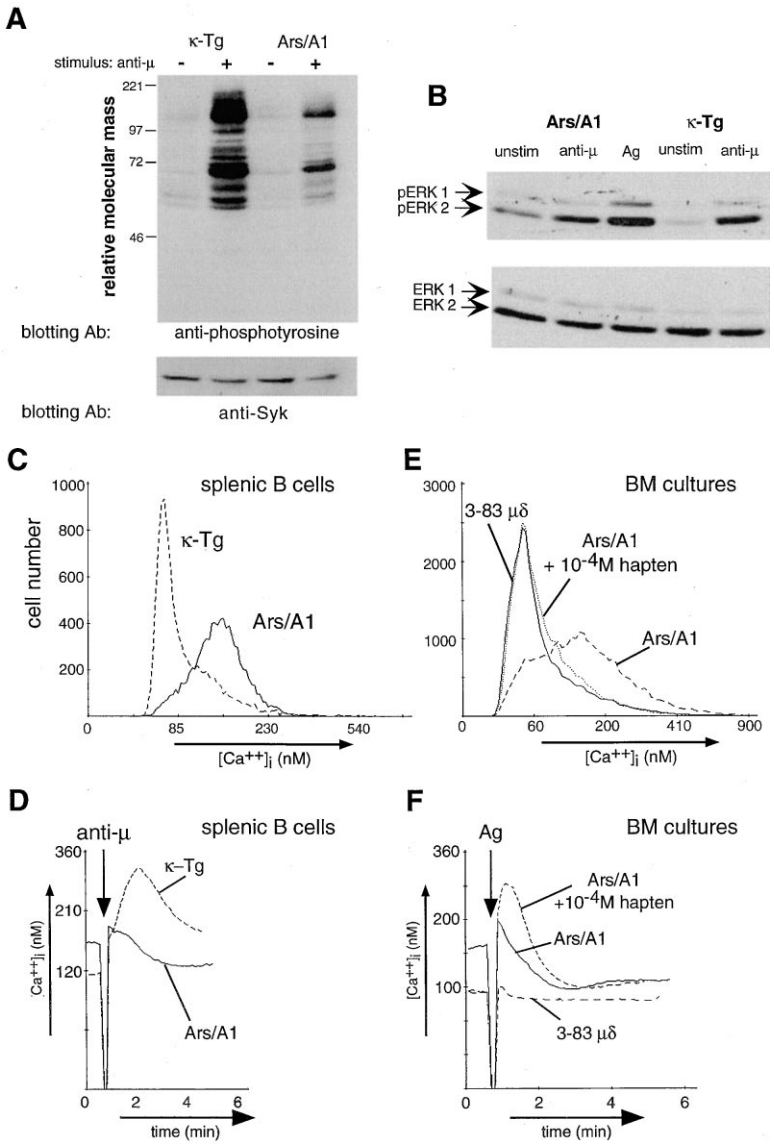


Figure 6. Ars/A1 B Cells Display an Anergic Signaling Phenotype

(A) Western blot analysis of stimulated (10 μg/ml anti-μ) versus unstimulated B cells ($2 \times 10^6$ cell equivalents per lane) showing tyrosine phosphorylation and Syk Western blots for quantitative control.

(B) Western blot analysis of phosphorylated Erk1 and Erk2 in stimulated (10 μg/ml anti-μ or 2 μg/ml antigen [$Ars_{14}$-BSA]) and unstimulated cells, as detected with phospho-Erk-specific antisera. Blots were stripped and reprobed using Erk1- and Erk2-specific antibodies.

(C and D) Splenic B cells were loaded with indo-1AM, and mean intracellular calcium concentration was evaluated using a flow cytometer. In (C), the distribution of $[Ca^{2+}]_i$ in Ars/A1 versus κ-Tg B cells is shown for unstimulated cells. In (D), kinetic analysis of $[Ca^{2+}]_i$ is shown following the addition of anti-μ (10 μg/ml).

(E and F) (E) Distribution of $[Ca^{2+}]_i$ and (F) calcium mobilization responses following addition of antigen (2 μg/ml $Ars_{14}$-BSA) were analyzed in immature B cells from Ars/A1 bone marrow (BM) cultures grown in medium alone or with the addition of Ars-tyr (see legend Figure 4). Cultured bone marrow from 3–83 μδ mice was analyzed similarly.

Based on the observations regarding marker expression by cultured bone marrow B cells from Ars/A1 mice (Figure 4), we analyzed the calcium signaling phenotype of these cells. The majority of cultured bone marrow cells exhibited high basal $[Ca^{2+}]_i$ unless they were propagated in the presence of free hapten (Figure 6E). Combined with the phenotypic analyses (Figure 4), these data show that at least some $IgM^{pos}IgD^{neg}$ cells display elevated baseline in $[Ca^{2+}]_i$, suggesting cells are stimulated at the immature stage. Cells propagated without free hapten failed to respond to BCR stimulation by antigen (Figure 6F) or anti-μ (data not shown). When Ars/A1 bone marrow cells were propagated in the presence of $10^{-4}$ M Ars-tyrosine, basal $[Ca^{2+}]_i$ was low (Figure 6E) and BCR aggregation led to a robust calcium response (Figure 6F). Control cells cultured from 3–83 μδ immunoglobulin Tg animals were included to demonstrate resting baseline $[Ca^{2+}]_i$ as well as the absence of nonspecific stimulation by the antigen. These data demonstrate that basal $[Ca^{2+}]_i$ is elevated and calcium mobilization responses are compromised in immature and transitional B cells from the Ars/A1 Tg animals. This is probably a consequence of BCR stimulation by endogenous antigen since the normal signaling phenotype can be restored if cells develop in the presence of free hapten.

## Discussion

Our results show that the BCR expressed by B cells of the Ars/A1 Tg mouse is specific not only for Ars but also for an unidentified autoantigen that is expressed in the bone marrow. Even though B cells in this animal populate peripheral lymphoid organs at normal frequencies, they exhibit an activated surface phenotype, with reduced levels of mIgM, and do not mount an immune response following immunization in vivo. Analysis of parameters of signal transduction demonstrates that Ars/A1 B cells exhibit high basal intracellular concentrations of $Ca^{2+}$, and BCR engagement with anti-μ, anti-idiotype,

or antigen fails to induce normal tyrosine phosphorylation and calcium mobilization. These observations indicate that an autoantigen induces a physiological state in these B cells that closely resembles or is equivalent to the anergic status of B cells in the HEL/anti-HEL tolerance model (Healy and Goodnow, 1998).

Our observations demonstrate that in the Ars/A1 model B cells encounter antigen at the immature stage, when they first express a mature BCR, in the bone marrow, similar to what was observed in the HEL/anti-HEL model (Mason et al., 1992). A significant portion of in vitro cultured Ars/A1 bone marrow B cells, while negative for IgD, display elevated levels of intracellular $Ca^{2+}$. This activation through the BCR appears to promote B cell development, as evidenced by increased IgD expression in vitro and by the virtual absence of transitional B cells in the spleen. Peripheral B cell development is an active process and requires signals through the BCR (Loder et al., 1999). It has been shown that influencing the strength of the signal can result in developmental arrest (e.g., $CD45^{-/-}$ [Loder et al., 1999]) or increased developmental progression (e.g., $SHIP^{-/-}$ [Brauweiler et al., 2000]). To further illustrate this, it was shown that while peripheral B cell development is blocked in anti-HEL Tg mice lacking CD45, additional expression of soluble HEL promotes accumulation of $IgD^{+}$ cells (Cyster et al., 1996). This lends supports to our data that suggests that augmentation of tonic signals through the BCR by low-level antigenic stimulation may enhance the rate of peripheral B cell development (Benschop and Cambier, 1999).

A major difference between the Ars/A1 mouse and the HEL/anti-HEL model is the affinity of the anergy-inducing autoantigen: while the affinity of the receptor for HEL is $\sim$2 nanomolar (Goodnow et al., 1988), several lines of evidence suggest that the Ars/A1 BCR binds its natural autoantigen with an affinity that is $>$10,000-fold lower. The best evidence for this is the observation that the activated phenotype in developing bone marrow B cells is almost completely precluded by monovalent Ars-tyrosine at a concentration of $10^{-4}$ M, despite the fact that the affinity of the Ars/A1 receptor for Ars is low at $\sim 2.5 \times 10^{5}\ M^{-1}$ (Sharon, 1990). At the same time, 50% inhibition of binding by this antibody to a multivalent form of Ars ($Ars_{14}$-BSA) requires a higher concentration of the monovalent hapten ($10^{-3}$ M). Thus, it is likely that the natural autoantigen has an affinity for the Ars/A1 BCR that is significantly less than $2.5 \times 10^{5}\ M^{-1}$.

Anti-Ars antibodies that are encoded by canonical H and $\kappa$ V region genes are a predominant feature of the strain A immune response to this hapten. Unmutated forms of this structure that are elicited in the early primary immune response only differ at two junctional codon positions in the H chain. By pairing an unmutated heavy chain from one antibody (mAb 36-65) with a heavily mutated light chain from another (mAb 36-71), it appears that we inadvertently generated an autoreactive binding site. An alternative view is that naturally occurring unmutated canonical B cells are slightly autoreactive and are capable of participating in an immune response under appropriate circumstances, for example, if provided with a timely source of activated T cell help (Bachmann et al., 1993; Goodnow et al., 1991; Cooke et al., 1994, 1998). Further experimentation is required to determine the precise biological capabilities of Tg B cells that are defined as "anergic" by accepted standards.

Although the Ars/A1 hybrid antibody binds ssDNA with low affinity, this is not necessarily the natural autoantigen that induces tolerance in Ars/A1 mice. ssDNA is unlikely to be stable in vivo, although it may be presented on dying cells (Casciola-Rosen et al., 1994). In addition, unmutated canonical antibodies such as mAb 36-65 also bind ssDNA with similar avidity (unpublished data), yet B cells expressing exact receptor correlates of these antibodies participate effectively in immune responses elicited by Ars, and they ultimately give rise to the most consistent and dominant component of the memory response. Thus, if ssDNA was the natural autoantigen responsible for the phenotype of Ars/A1 B cells, this would imply that naturally occurring unmutated canonical B cells have a similar phenotype but they are nonetheless capable of participating in immune reactions under appropriate circumstances.

Such a scenario, wherein generated autoreactive B cells must be silenced, has been put forward to explain the existence of anergic B cells (Goodnow, 1996; Goodnow et al., 1991). Indeed, studies in $\mu$ H-chain Tg mice demonstrated differences between ssDNA- and dsDNA-reactive B cells with respect to developmental arrest and their ability to be stimulated through the BCR (Mandik-Nayak et al., 1997; Roark et al., 1997; Noorchashm et al., 1999). It was also demonstrated that severely anergic anti-dsDNA B cells can be rescued by providing T cell signals in vitro (anti-CD40 and IL-4 [Noorchashm et al., 1999]), giving credence to the idea that anergy in autoreactive B cells can be reversed under certain circumstances. Antibodies that bind DNA, however, often have secondary specificities for other autoantigens such as cardiolipin and Sm (Diamond and Scharff, 1984; Retter et al., 1996; Santulli-Marotto et al., 1998; Takeda et al., 1999; Eivazova et al., 2000) so it is plausible that the Ars/A1 receptor binds another autoantigen in addition to ssDNA.

It is interesting that we find no evidence of receptor editing in Ars/A1 bone marrow B cells. A similar observation has been reported in mice expressing an immunoglobulin $\mu$ Tg that encodes a BCR with affinity for ssDNA (Erikson et al., 1991). B cells in this mouse, as in the Ars/A1 mouse, are functionally silenced in vivo but, in contrast to the Ars/A1 B cells, express high levels of IgM (Erikson et al., 1991). Interestingly, when the affinity of the Tg receptor for ssDNA is increased, B cells are deleted or edited in the bone marrow (Chen et al., 1994, 1995). Based on this observation and differences in tolerance mechanisms in the HEL model when HEL is secreted or membrane bound (Goodnow et al., 1988; Hartley et al., 1991), it appears that the degree of BCR aggregation is one of the variables that determines the pathway of tolerance. This would explain the findings that in HEL/anti-HEL double-Tg mice immature BM B cells carry HEL on their receptors, resulting in anergy in the apparent absence of receptor editing (Mason et al., 1992). In contrast to the Ars/A1 model, HEL binding at that stage resulted in downmodulation of IgM. It is likely that in the Ars/A1 mouse, BCR aggregation is modest due to affinity/valence considerations, and this could be a major factor that determines their anergic fate.

Collectively, our data support a scenario wherein Ars/A1 B cells encounter antigen at the immature stage in the bone marrow. Correlated temporally with movement to the transitional stage, they upregulate CD69, CD80, and CD86. Since there are (apparently) no appropriate T helper cells in this environment, they fail to make productive contact. These transitional B cells continue to develop and leave the bone marrow. By the time they reach the spleen, surface CD80 and particularly CD86 have returned to nearly basal levels that may be inadequate for productive interactions with T cells even if they encounter T cells that recognize class II MHC-peptide complexes they present. In fact, one might imagine that these B cells should induce tolerance in T cells. We hypothesize that B cell anergy in this model may be the consequence of B cell stimulation by antigen, with consequent transient upregulation of T cell costimulatory molecules and receptor desensitization, followed by a failed search for T cell help. In this condition, the B cell, expressing too little CD80 or CD86 to elicit helper activity from resting T cells and unable to respond again to antigen, is destined to die a failure. Appropriate T cell help is crucial, and it has been shown that anergic B cells can be rescued by timely T cell help in vivo (Goodnow et al., 1991; Bachmann et al., 1993; Cooke et al., 1994; Cook et al., 1998). Future experiments will address whether Ars/A1 B cells can be rescued by T cell help at the transitional stage in the bone marrow.

We believe that the Ars/A1 model provides a good approximation of a natural tolerance scenario because the autoantigen binds the Tg BCR with low affinity. It is likely that most autoreactive B cells arising from the bone marrow bind self with low affinity based on first principles. For example, most primary immune responses are composed of antibodies with low affinity for immunogen, despite the enhancing power of affinity selection. In addition, random pairs of H and L chain V region genes displayed in phage libraries generally yield low affinity products upon an initial screen. Mutagenesis and further rounds of selection applied artificially in vitro or naturally in vivo are required to achieve antibodies of high affinity. The capacity of monovalent hapten to block the appearance of the anergic phenotype in developing bone marrow B cells is an important element of this model. Ars-tyrosine, present during the IL-7 bone marrow cultures, precluded development of cells with an anergic phenotype. Thus, the power of this model may ultimately derive from both its emulation of physiological affinity receptor-antigen interaction and the ability to control B cell perception of an autoantigen in both quantitative and temporal terms.

## Experimental Procedures

### Generation of IgH- and IgL-Chain Tg Mice

Heavy chain: a ~50 kb cosmid construct with a VH-D-JH exon from a prototypic canonical anti-Ars antibody (36–65 [Siekevitz et al., 1983]) in the context of complete $\mu$ and $\delta$ constant region genes was generated by first replacing the VH-D-JH exon in a previously described genomic $\mu$ gene (Durdik et al., 1989) with the corresponding exon from hybridoma 36–65. This construct was linearized at a 3′-proximal Xho I site. It was then ligated to a similarly linearized 3–83 $\mu\delta$ cosmid construct (Russell et al., 1991) and encapsidated with a phage $\lambda$ packing extract to produce the desired heavy chain cosmid construct, which was then freed of plasmid-derived sequences by NruI digestion before injection (Figure 1A). The construct contained a natural promoter for the VH gene and the natural $\mu$-intron enhancer. The VH-D-JH exon carried no somatic mutations producing amino acid replacements. FVB-derived eggs were injected with this construct together with a segment of DNA containing the *Igh* 3′ enhancer (HS1,2,3b,4 [Madisen and Groudine, 1994]) to produce a line carrying one copy of the H chain transgene. Preservation of the original V region sequences in the Tg mouse was confirmed by PCR amplification and sequencing. H chain transgenic animals were backcrossed for two to three generations onto A/J strain mice.

Light chain: a 14 kb construct containing a functionally rearranged $\kappa$ light chain from another canonical anti-Ars antibody, mAb36-71, together with natural intron and 3′ enhancers was generated (Figure 1A) and injected into B6 eggs to produce a second Tg mouse line carrying three to four copies of the transgene. The V$\kappa$-J$\kappa$ exon in this construct carried 11 amino acid replacements that were natural consequences of somatic mutagenesis (Sharon et al., 1989). A B6 mouse homozygous for the mutant $\kappa$ Tg was bred to the heterozygous $\mu\delta$ chain Tg mouse to generate $\mu\delta + \kappa$ double Tg mice, which we refer to as Ars/A1, and $\kappa$-only Tg littermate controls.

### Cells and Tissue Culture

Splenic B cells ($\rho > 1.066$) were prepared as previously described (Vilen et al., 1997) from 2- to 3-month-old adult mice. Cell viability was assessed using trypan blue dye exclusion. Immature B cells were obtained from IL-7-driven bone marrow cultures as described in detail elsewhere (Benschop et al., 1999).

### Immunization, Serology, and Immunohistochemistry

Mice (8–12 weeks old) were immunized by a single intraperitoneal injection of 100 $\mu$g $Ars_{14}$-KLH in 200 $\mu$l PBS in incomplete Freunds' adjuvant. Pre- (day 0) and post- (day 13) immunization sera were analyzed for Ars-specific antibody titers using ELISA. Plates were coated for 6 hr at room temperature with 1 $\mu$g/well $Ars_{14}$-BSA and blocked with 2%BSA/1%gelatin in PBS for 1 hr at room temperature. Serial dilutions of sera and standards were added and plates were incubated overnight at 4°C. The assays were developed with biotin-labeled rat-anti-mouse $\kappa$ (clone 187.1, ATCC) and SA-HRP (Southern Biotechnology, Birmingham, AL). Between steps, plates were washed copiously with PBS and developed with ABTS for colorimetric detection. On day 13, the mice were sacrificed. Spleens were embedded in Tissue-Tek O.C.T. (Fisher Scientific, Pittsburgh, PA), frozen in dry ice–cooled 2-methylbutane, and stored at −70°C until sectioning. Spleens were cut on a cryostat and successive sections of 6–8 $\mu$m were thaw-mounted on slides. Sections were air-dried, acetone-fixed for 5 min, and stored at −70°C. After thawing, slides were rehydrated with 5% normal goat serum in PBS (10 min), and the spleens were stained in a humidified chamber for 30 min at room temperature. The reagents used were PNA-biotin (Vector Laboratories, Burlingame, CA) and anti-idiotype-biotin (5Ci [Wysocki and Sato, 1981]). After three washes with PBS, the sections were developed with the ABC Elite Kit followed by DAB detection (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocols. After counterstaining with hematoxilin, the slides were mounted with Permount. Sections were photographed using a Nikon Diaphot coupled to a Photometrix CCD camera and analyzed using IPLab Spectrum Version 3.1a. Germinal center areas were measured; 98% of the GCs were in the range of 0–360 pixels.

### Phenotypic Analysis

Cells were resuspended in PBS containing 1% BSA and 0.1% sodium azide and incubated with an optimal amount of biotinylated or directly fluorescinated antibody or antigen ($Ars_8$-BSA). Antibodies directed against the following molecules were used: IgMa (RS3.1, ATCC); IgMb (AF6-78, BD Pharmingen, San Diego, CA); kappa (187.1, ATCC); lambda (polyclonal, Southern Biotechnology, Birmingham, AL); CD69 (H1.2F3, BD Pharmingen, San Diego, CA); CD86 (GL-1, BD Pharmingen, San Diego, CA); CD80 (16-10A1, BD Pharmingen, San Diego, CA); CD21/CD35 (CR2/CR1; 7G6, BD Pharmingen, San Diego, CA); CD19 (1D3, BD Pharmingen, San Diego, CA); IgM (b-7-6); IgD (JA12.5) or polyclonal (Southern Biotechnology, Birmingham, AL); CD22 (CY34.1.2, ATTC); CD23 (IgE Fc receptor;

B3B4, BD Pharmingen, San Diego, CA); CD45R (anti-B220; RA3-6B2, ATCC); E4 (anti-idiotype [Wysocki and Sato, 1981]); CD62L (MEL-14, ATCC). Cells were incubated for 30 min at 4°C and washed twice in PBS/BSA/azide. In the case of biotinylated reagents, cells were incubated as before with streptavidin-FITC or -TC (Caltag, Burlingame, CA). After washing, cells were analyzed on a flow cytometer. Cells were gated on light scatter profile, and dead cells were excluded using 7AAD stain (Via-Probe, BD Pharmingen, San Diego, CA).

### Calcium Mobilization

For measurements of free intracellular calcium concentration ($[Ca^{2+}]_i$), cells were loaded with Indo-1AM (Molecular Probes, Eugene, OR), labeled with anti-B220-PE, suspended at $10^6$ cells/ml in IMDM, and analysis initiated using flow cytometry. After the baseline was established, cells were stimulated with either antigen or anti-μ antibody (b-7-6). Mean $[Ca^{2+}]_i$ was evaluated over time using a MoFlo cytometer (Cytomation, Ft. Collins, CO) with appended data acquisition system, and MultiTime software (Phoenix Flow Systems, San Diego, CA) was used for analysis.

### Immunoblotting

Cells were lysed in 1% NP-40 lysis buffer (150 mM NaCl, 10 mM Tris [pH = 7.5], 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM PMSF, 10 mM NaF, 0.4 mM EDTA, 1 mM aprotinin, 1 mM α-1-antitrypsin, and 1 mM leupeptin). Lysates were kept on ice for 15 min before centrifugation at 14,000 rpm for 10 min in an Eppendorf centrifuge. Supernatants were mixed with SDS reducing sample buffer and boiled for 5 min. Proteins were separated by SDS-PAGE, transferred to PVDF, and visualized using specific antibodies followed by enhanced chemiluminescence (NEN, Boston, MA). To detect tyrosine phosphorylation, the AB-2 anti-phospho-tyrosine antibody was used (Oncogene, Boston, MA); polyclonal rabbit anti-Syk was prepared in our laboratory; anti-phospho Erk1/2 was obtained from New England Biolabs (Beverly, MA) and used to assess Erk1/2 phosphorylation, anti-Erk1 and Erk2 were from Santa Cruz (Santa Cruz, CA).

### Analysis for Recombinase Activator Gene-2 Expression

Levels of recombinase activator gene-2 (RAG-2) and GαS mRNA were determined by RT-PCR assay as described in detail elsewhere (Hertz and Nemazee, 1997; Melamed et al., 1997).

### ssDNA Binding and Hapten Competition Assays

Plates were precoated with 0.1% poly-L-lysine overnight in water. ssDNA (10 μg/ml) or $Ars_{14}$-BSA (10 μg/ml) was added and incubated for 1 hr. Plates were incubated with 400 ng antibody/well in the presence or absence of various concentrations of hapten-tyrosine. Antibody binding was detected using $^{125}$I-Rat anti-mouse κ antibody (clone 187.1, ATCC). The amino group of tyrosine was blocked by acetylation prior to conjugation with diazonium salts of Ars and *p*-aminobenzoic acid-tyrosine (PABA [Wysocki and Sato, 1981]).

### Acknowledgments

We thank Erin Brandl and William Townend for technical assistance, Chris Snyder for typing and maintaining Ars/A1 Tg mice, Gary Shapiro for allotype analyses, and Jacqueline Sharon for providing transfectants expressing an IgG2b correlate of the Ars/A1 antibody. These studies were supported by grants from the National Institutes of Health awarded to J. C. C. (P1AI22295, AG13983, AI20519), L. J. W. (AI33613, P1AI22295), and T. M. (AI38965). J. C. C. is an Ida and Cecil Green Professor of Cell Biology, and R. J. B. is recipient of a Leukemia and Lymphoma Society Special Fellow Award.

Received August 9, 2000; revised December 20, 2000.

### References

Bachmann, M.F., Rohrer, U.H., Kundig, T.M., Burki, K., Hengartner, H., and Zinkernagel, R.M. (1993). The influence of antigen organization on B cell responsiveness. Science *262*, 1448–1451.

Benschop, R.J., and Cambier, J.C. (1999). B cell development: signal transduction by antigen receptors and their surrogates. Curr. Opin. Immunol. *11*, 143–151.

Benschop, R.J., Melamed, D., Nemazee, D., and Cambier, J.C. (1999). Distinct signal thresholds for the unique antigen receptor-linked gene expression programs in mature and immature B cells. J. Exp. Med. *190*, 749–756.

Brauweiler, A., Tamir, I., Dal Porto, J., Benschop, R.J., Helgason, C.D., Humphries, R.K., Freed, J.H., and Cambier, J.C. (2000). Differential regulation of B cell development, activation, and death by the src homology 2 domain-containing 5′ inositol phosphatase (SHIP). J. Exp. Med. *191*, 1545–1554.

Cambier, J.C., Kettman, J.R., Vitetta, E.S., and Uhr, J.W. (1976). Differential susceptibility of neonatal and adult murine spleen cells to in vitro induction of B-cell tolerance. J. Exp. Med. *144*, 293–297.

Carsetti, R., Kohler, G., and Lamers, M.C. (1995). Transitional B cells are the target of negative selection in the B cell compartment. J. Exp. Med. *181*, 2129–2140.

Casciola-Rosen, L.A., Anhalt, G., and Rosen, A. (1994). Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. J. Exp. Med. *179*, 1317–1330.

Chen, C., Radic, M.Z., Erikson, J., Camper, S.A., Litwin, S., Hardy, R.R., and Weigert, M. (1994). Deletion and editing of B cells that express antibodies to DNA. J. Immunol. *152*, 1970–1982.

Chen, C., Nagy, Z., Radic, M.Z., Hardy, R.R., Huszar, D., Camper, S.A., and Weigert, M. (1995). The site and stage of anti-DNA B-cell deletion. Nature *373*, 252–255.

Cook, M.C., Basten, A., and Fazekas de St. Groth, B. (1998). Rescue of self-reactive B cells by provision of T cell help in vivo. Eur. J. Immunol. *28*, 2549–2558.

Cooke, M.P., Heath, A.W., Shokat, K.M., Zeng, Y., Finkelman, F.D., Linsley, P.S., Howard, M., and Goodnow, C.C. (1994). Immunoglobulin signal transduction guides the specificity of B cell-T cell interactions and is blocked in tolerant self-reactive B cells. J. Exp. Med. *179*, 425–438.

Cyster, J.G., Healy, J.I., Kishihara, K., Mak, T.W., Thomas, M.L., and Goodnow, C.C. (1996). Regulation of B-lymphocyte negative and positive selection by tyrosine phosphatase CD45. Nature *381*, 325–328.

Diamond, B., and Scharff, M.D. (1984). Somatic mutation of the T15 heavy chain gives rise to an antibody with autoantibody specificity. Proc. Natl. Acad. Sci. USA *81*, 5841–5844.

Durdik, J., Gerstein, R.M., Rath, S., Robbins, P.F., Nisonoff, A., and Selsing, E. (1989). Isotype switching by a microinjected mu immunoglobulin heavy chain gene in transgenic mice. Proc. Natl. Acad. Sci. USA *86*, 2346–2350.

Eivazova, E.R., McDonnell, J.M., Sutton, B.J., and Staines, N.A. (2000). Cross-reactivity of antiidiotypic antibodies with DNA in systemic lupus erythematosus. Arthritis Rheum. *43*, 429–439.

Erikson, J., Radic, M.Z., Camper, S.A., Hardy, R.R., Carmack, C., and Weigert, M. (1991). Expression of anti-DNA immunoglobulin transgenes in non-autoimmune mice. Nature *349*, 331–334.

Eris, J.M., Basten, A., Brink, R., Doherty, K., Kehry, M.R., and Hodgkin, P.D. (1994). Anergic self-reactive B cells present self antigen and respond normally to CD40-dependent T-cell signals but are defective in antigen-receptor-mediated functions. Proc. Natl. Acad. Sci. USA *91*, 4392–4396.

Gay, D., Saunders, T., Camper, S., and Weigert, M. (1993). Receptor editing: an approach by autoreactive B cells to escape tolerance. J. Exp. Med. *177*, 999–1008.

Goodnow, C.C. (1996). Balancing immunity and tolerance: deleting and tuning lymphocyte repertoires. Proc. Natl. Acad. Sci. USA *93*, 2264–2271.

Goodnow, C.C., Crosbie, J., Adelstein, S., Lavoie, T.B., Smith-Gill, S.J., Brink, R.A., Pritchard-Briscoe, H., Wotherspoon, J.S., Loblay, R.H., Raphael, K., et al. (1988). Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. Nature *334*, 676–682.

Goodnow, C.C., Brink, R., and Adams, E. (1991). Breakdown of self-tolerance in anergic B lymphocytes. Nature *352*, 532–536.

Hartley, S.B., Crosbie, J., Brink, R., Kantor, A.B., Basten, A., and Goodnow, C.C. (1991). Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. Nature *353*, 765–769.

Hathcock, K.S., Laszlo, G., Pucillo, C., Linsley, P., and Hodes, R.J. (1994). Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. J. Exp. Med. *180*, 631–640.

Healy, J.I., and Goodnow, C.C. (1998). Positive versus negative signaling by lymphocyte antigen receptors. Annu. Rev. Immunol. *16*, 645–670.

Healy, J.I., Dolmetsch, R.E., Timmerman, L.A., Cyster, J.G., Thomas, M.L., Crabtree, G.R., Lewis, R.S., and Goodnow, C.C. (1997). Different nuclear signals are activated by the B cell receptor during positive versus negative signaling. Immunity *6*, 419–428.

Hertz, M., and Nemazee, D. (1997). BCR ligation induces receptor editing in $IgM^{+}IgD^{-}$ bone marrow B cells in vitro. Immunity *6*, 429–436.

Kuettner, M.G., Wang, A.L., and Nisonoff, A. (1972). Quantitative investigations of idiotypic antibodies. VI. Idiotypic specificity as a potential genetic marker for the variable regions of mouse immunoglobulin polypeptide chains. J. Exp. Med. *135*, 579–595.

Leo, O., Slaoui, M., Marvel, J., Milner, E.C., Hiernaux, J., Moser, M., Capra, J.D., and Urbain, J. (1985). Idiotypic analysis of polyclonal and monoclonal anti-p-azophenylarsonate antibodies of BALB/c mice expressing the major cross- reactive idiotype of the A/J strain. J. Immunol. *134*, 1734–1739.

Liu, A.H., Jena, P.K., and Wysocki, L.J. (1996). Tracing the development of single memory-lineage B cells in a highly defined immune response. J. Exp. Med. *183*, 2053–2063.

Loder, F., Mutschler, B., Ray, R.J., Paige, C.J., Sideras, P., Torres, R., Lamers, M.C., and Carsetti, R. (1999). B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. J. Exp. Med. *190*, 75–89.

Madisen, L., and Groudine, M. (1994). Identification of a locus control region in the immunoglobulin heavy- chain locus that deregulates c-myc expression in plasmacytoma and Burkitt's lymphoma cells. Genes Dev. *8*, 2212–2226.

Mandik-Nayak, L., Bui, A., Noorchashm, H., Eaton, A., and Erikson, J. (1997). Regulation of anti-double-stranded DNA B cells in nonautoimmune mice: localization to the T-B interface of the splenic follicle. J. Exp. Med. *186*, 1257–1267.

Manser, T., Tumas-Brundage, K.M., Casson, L.P., Giusti, A.M., Hande, S., Notidis, E., and Vora, K.A. (1998). The roles of antibody variable region hypermutation and selection in the development of the memory B-cell compartment. Immunol. Rev. *162*, 183–196.

Mason, D.Y., Jones, M., and Goodnow, C.C. (1992). Development and follicular localization of tolerant B lymphocytes in lysozyme/anti-lysozyme IgM/IgD transgenic mice. Int. Immunol. *4*, 163–175.

Melamed, D., Kench, J.A., Grabstein, K., Rolink, A., and Nemazee, D. (1997). A functional B cell receptor transgene allows efficient IL-7-independent maturation of B cell precursors. J. Immunol. *159*, 1233–1239.

Melamed, D., Benschop, R.J., Cambier, J.C., and Nemazee, D. (1998). Developmental regulation of B lymphocyte immune tolerance compartmentalizes clonal selection from receptor selection. Cell *92*, 173–182.

Metcalf, E.S., and Klinman, N.R. (1976). In vitro tolerance induction of neonatal murine B cells. J. Exp. Med. *143*, 1327–1340.

Nemazee, D. (2000). Receptor editing in B cells. Adv. Immunol. *74*, 89–126.

Nemazee, D.A., and Burki, K. (1989). Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. Nature *337*, 562–566.

Nemazee, D., Kouskoff, V., Hertz, M., Lang, J., Melamed, D., Pape, K., and Retter, M. (2000). B-cell-receptor-dependent positive and negative selection in immature B cells. Curr. Top. Microbiol. Immunol. *245*, 57–71.

Noorchashm, H., Bui, A., Li, H.L., Eaton, A., Mandik-Nayak, L., Sokol, C., Potts, K.M., Pure, E., and Erikson, J. (1999). Characterization of anergic anti-DNA B cells: B cell anergy is a T cell- independent and potentially reversible process. Int. Immunol. *11*, 765–776.

Norvell, A., Mandik, L., and Monroe, J.G. (1995). Engagement of the antigen-receptor on immature murine B lymphocytes results in death by apoptosis. J. Immunol. *154*, 4404–4413.

Pike, B.L., Boyd, A.W., and Nossal, G.J. (1982). Clonal anergy: the universally anergic B lymphocyte. Proc. Natl. Acad. Sci. USA *79*, 2013–2017.

Retter, M.W., Cohen, P.L., Eisenberg, R.A., and Clarke, S.H. (1996). Both Sm and DNA are selecting antigens in the anti-Sm B cell response in autoimmune MRL/lpr mice. J. Immunol. *156*, 1296–1306.

Roark, J.H., Bui, A., Nguyen, K.A., Mandik, L., and Erikson, J. (1997). Persistence of functionally compromised anti-double-stranded DNA B cells in the periphery of non-autoimmune mice. Int. Immunol. *9*, 1615–1626.

Rolink, A., Kudo, A., Karasuyama, H., Kikuchi, Y., and Melchers, F. (1991). Long-term proliferating early pre B cell lines and clones with the potential to develop to surface Ig-positive, mitogen reactive B cells in vitro and in vivo. EMBO J. *10*, 327–336.

Russell, D.M., Dembic, Z., Morahan, G., Miller, J.F., Burki, K., and Nemazee, D. (1991). Peripheral deletion of self-reactive B cells. Nature *354*, 308–311.

Santulli-Marotto, S., Retter, M.W., Gee, R., Mamula, M.J., and Clarke, S.H. (1998). Autoreactive B cell regulation: peripheral induction of developmental arrest by lupus-associated autoantigens. Immunity *8*, 209–219.

Sharon, J. (1990). Structural correlates of high antibody affinity: three engineered amino acid substitutions can increase the affinity of an anti-p-azophenylarsonate antibody 200-fold. Proc. Natl. Acad. Sci. USA *87*, 4814–4817.

Sharon, J., Gefter, M.L., Wysocki, L.J., and Margolies, M.N. (1989). Recurrent somatic mutations in mouse antibodies to p-azophenylarsonate increase affinity for hapten. J. Immunol. *142*, 596–601.

Siekevitz, M., Huang, S.Y., and Gefter, M.L. (1983). The genetic basis of antibody production: a single heavy chain variable region gene encodes all molecules bearing the dominant anti-arsonate idiotype in the strain A mouse. Eur. J. Immunol. *13*, 123–132.

Takeda, I., Rayno, K., Wolfson-Reichlin, M., and Reichlin, M. (1999). Heterogeneity of anti-dsDNA antibodies in their cross-reaction with ribosomal P protein. J. Autoimmun. *13*, 423–428.

Tiegs, S.L., Russell, D.M., and Nemazee, D. (1993). Receptor editing in self-reactive bone marrow B cells. J. Exp. Med. *177*, 1009–1020.

Vilen, B.J., Famiglietti, S.J., Carbone, A.M., Kay, B.K., and Cambier, J.C. (1997). B cell antigen receptor desensitization: disruption of receptor coupling to tyrosine kinase activation. J. Immunol. *159*, 231–243.

Wysocki, L.J., and Sato, V.L. (1981). The strain A anti-p-azophenylarsonate major cross-reactive idiotypic family includes members with no reactivity toward p-azophenylarsonate. Eur. J. Immunol. *11*, 832–839.

Wysocki, L.J., Liu, A.H., and Jena, P.K. (1998). Somatic mutagenesis and evolution of memory B cells. Curr. Top. Microbiol. Immunol. *229*, 105–131.